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A Subpopulation of RNA3 of *Cucumber mosaic virus* Quasispecies

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This study examined the existence of genetically diverse population of *Cucumber mosaic virus* (CMV), known as quasispecies, from lily, *Nicotiana benthamiana* and from purified virions. Based on the conserved sequences of CMV lily isolates in intergenic region (IR) on RNA3, the genetic variation of IR from three different sources was investigated by a specific restriction endonuclease hydrolysis of amplified reverse transcription-polymerase chain reaction (RT-PCR) products using virus-specific primers, and was compared with IR sequences. The IR nucleotide sequences of CMV lily isolates were highly conserved, however, quasispecies was detected from all three sources in low level, containing subpopulations of RNA3. These subpopulations of RNA3 were inoculated onto zucchini squash by *in vitro* transcripts from corresponding full-length cDNA clones together with Fny RNA1 and 2 transcripts. The systemic symptom of zucchini plants infected by these quasispecies was chlorotic spotting, which was milder than severe mosaic and stunt symptom caused by Fny-CMV. The severity of symptom was correlated with RNA accumulation of viruses. These results suggest that the genome of CMV lily isolates consists of quasispecies populations.

Keywords **:** *Cucumber mosaic virus*, intergenic region, lily, quasispecies, subpopulation.

Viruses have extreme evolutionary capacities that have allowed adaptation to parasitize numerous host species. Among them, plant viruses have evolved into genetically diverse populations referred to as quasispecies, by means of genetic mode of RNA genome associated with error-prone replication, rapid rate of replication, and large population (Domingo et al., 2002; Rodriguez-Alvarado et al., 1995). Plant viral quasispecies are dynamic and complex distributions that react to environmental changes, resulting in a pool of mutants which could become a selective advantaged RNA species to new environment (Domingo et al., 1996; Roossinck and Palukaitis, 1990). Many reports suggested that the population diversity of *Cucumber mosaic virus* (CMV) has very different levels of variations in common hosts and commercial crops (Aranda et al., 1993; Kurath and Palukaitis, 1989; Palukaitis and Roossinck, 1995; Rodriguez-Alvarado et al., 1995; Schneider and Roossinck, 2001).

CMV, the type species of the genus *Cucumovirus* in the family *Bromoviridae*, contains a tripartite genome of messenger-sense single-stranded RNA, designated as RNAs 1, 2 and 3 in order of decreasing size (Palukaitis et al., 1992). RNA3 encodes for two proteins involved in viral movement and encapsidation (Canto et al., 1997). Meanwhile, RNA2 encodes for the 2a protein, which is a RNA-dependent RNA polymerase of replication complex, whereas, RNA1 encodes for the 1a protein, another subunit of CMV replicase complex (Hayes and Buck, 1990). CMV has the largest host range encompassing over 1000 species of plants in 365 genera of 85 families (Palukaitis et al., 1992). Also, it has been suggested that a few CMV isolates have shown different biological and genetic properties from typical CMV strains. In particular, some CMV isolates from lily and alstroemeria have these variant properties. Chen et al. (2002) demonstrated that CMV subgroup II isolates from alstroemeria contained additional sequences of various lengths in the 3' noncoding region (NCR) that were generated by RNA recombination. In addition, some reports on lily isolates of CMV showed that most lily isolates could not infect tobacco plants and cucurbits unlike the typical CMV strains, but the genome of lily isolates was highly homologous (Chen et al., 2001; Jung et al., 2000; Masuta et al., 2002; Ryu et al., 2002). This report shows that RNA3 of CMV lily isolates exists in the subpopulation.

Materials and Methods

Virus source and reverse transcription-polymerase chain reaction. To identify the existence of quasispecies, Korean CMV lily isolates, designated as LK, LK4, LK6, LK7, and LK8, were all independently isolated from cultivated lily plants in Korea (Table 1). All CMV isolates were maintained on *Nicotiana*

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Table 1. Isolation host and total number of quasispecies for CMV lily isolates analyzed in viral populations derived from three different sources

Isolate	Source			Isolation host
	Lily[a]	*N. benthamiana*[b]	Purified virions	
LK	10[c]/90[d]	4/96	5/95	*Lilium* Oriental hybrid cv. Marco polo
LK4	9/91	3/97	4/96	*Lilium* Oriental hybrid cv. Casa Blanca
LK6	11/89	5/95	5/95	*Lilium lancifolium*
LK7	8/92	4/96	5/95	*Lilium maculatum*
LK8	10/90	4/96	6/94	*Lilium longiflorum*

[a] The leaves of naturally infected lily plants.
[b] The systemic leaves of *N. benthamiana* infected by each CMV isolate.
[c] The number of undigested clones by *Bam*HI.
[d] The number of digested clones by *Bam*HI.

benthamiana by mechanical inoculation from diseased lily plants, and subsequently, virus purification of each isolate was performed by previous protocol (Peden and Symons, 1973). The total RNAs from individual lily plants and upper leaves of *N. benthamiana,* and purified viral RNAs from virions multiplied in *N. benthamiana* were used as templates in high-fidelity reverse transcription-polymerase chain reaction (RT-PCR). RT reaction was performed at 42°C for 60 minutes with CMV-CP-reverse primer (corresponding to nt 1286 to 1313 of Fny RNA3). PCR was carried out using Expand 20Kb^{PLUS} PCR system (Roche) included in proof-reading polymerase with CMV-3a-forward primer (corresponding to nt 120 to 147 of Fny RNA3) and reverse primer (Fig. 1) under the conditions of 94°C, 2 minutes (1 cycle), 94°C, 30 seconds, 48°C, 30 seconds, 72°C, 60 seconds (25 cycles), 72°C, 5 minutes. To select the quasispecies, each RT-PCR product was digested by *Bam*HI-digestion. Also, the synthesized RT-PCR products were directly cloned into pGEM-T easy vector (Promega). The proportion of quasispecies was analyzed from random selected clones, according to the digestion of *Bam*HI. The nucleotide sequences were determined by automated sequencing protocol using ABI 377 sequencer from random selection of clones (Sanger et al., 1977) and analyzed by DNASTAR package software (Lasergene).

Construction of full-length cDNA of RNA3 and symptom analysis. To obtain the full-length cDNA of quasispecies RNA3, the viral RNA from purified virus particles of LK-CMV was used. RT was performed at 42°C for 60 minutes in a 50 μL reaction mixture containing 1 μL (ca. 100 ng) of purified viral RNA (preheating at 65°C for 10 minutes and chilling on ice for 5 minutes), 10 μL 5x reaction buffer, 100 ng of reverse primer (5'-AATT**CTGCAG**TGGTCTCCTTTTRGAGGCC-3') with PstI site (in bold) and 19 nt identical to those at 3' end of Fny-CMV RNA3, 10 mM dithiothreitol, 1 mM deoxynucleoside triphosphates (dNTP), 10 units of RNase inhibitor (Promega), and 400 units of Superscript II RNase H$^-$ reverse transcriptase (Invitrogen). PCR was carried out by using forward primer (5'-AATC**GAATTC**TAATACGACTCACTATAGTAATCTTACC ACTGTGTGT-3') with EcoRI (in bold), T7 RNA polymerase promoter (underlined), and 20 nt identical to those at 5' end of Fny RNA3 and a reverse primer in 50 μL reaction volume containing 5 μL RT solution, 5 μL of 10x Expand 20Kb^{PLUS} PCR buffer (Roche) containing 22.5 mM $MgCl_2$, 10 ng of forward primer as described above, 10 ng of reverse primer, 1 mM dNTP, and 2.5 units of Expand 20Kb^{PLUS} Polymerase Mixture (Roche) containing a proof-reading polymerase in a programmable DNA thermer cycler (iCycler, Bio-Rad). The condition of PCR was 94°C, 2 minutes (1 cycle); 94°C, 20 seconds, 55°C, 30 seconds, 68°C, 3 minutes (5 cycles); 94°C, 20 seconds, 57°C, 30 seconds,

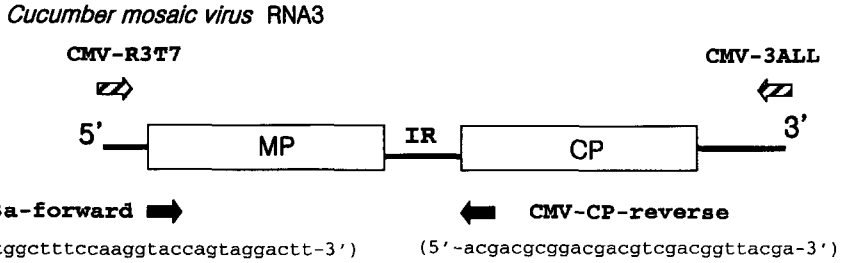

Fig. 1. Schematic representation of the RNA3 genome of *Cucumber mosaic virus* (CMV) showing the location of two pairs of RT-PCR primers used in this study. The pair of primers, designated as CMV-3a-forward and CMV-CP-reverse, conducted to analyze the quasispecies of LK-CMV is represented by the closed arrow. The portion of viral genome encoding the movement protein (MP), intergenic region (IR), and 5'-flanking region of coat protein (CP) gene was amplified and cloned for sequence analysis. A primer set, named CMV-R3T7 and CMV-3ALL, was used to construct full-length cDNA of LK-CMV RNA3. The open boxes represent ORFs while the terminal lines represent the 5' and 3' untranslational regions.

68°C, 3 minutes (25 cycles).

Synthesized cDNAs were hydrolyzed with *Eco*RI-*Pst*I enzymes, and the fragment was purified from agarose gel using QIAquick Gel Extraction Kit (QUIAGEN) based on the manufacturer's instructions. The purified cDNA of LK-CMV was ligated into pUC18 digested with the same restriction endonucleases (Fig. 1). Each cDNA clone of RNA3s was selected by *Bam*HI digestion, and named as pLK3 and pLK-noB, respectively.

To examine the biological properties of quasispecies viruses, pseudorecombination test was performed on zucchini squash as model plant. RNA3 pseudorecombinant CMVs, FFL and FFL-noB, were generated from each clone together with RNA1 and 2 of Fny-CMV (Rizzo and Palukaitis, 1990). The clones were linearized at the 3' end of the inserted sequence using *Pst*I, blunt-ended. The RNA3 transcripts were obtained from *in vitro* transcription system with T7 RNA polymerase (Promega) in the presence of cap analog m^{7}GpppG (New England Biolabs), and mixed with RNA1 and 2 transcripts generated from pFny109 and pFny209 (Rizzo and Palukaitis, 1990), as described previously (Canto et al., 2001). Pseudorecombinant viruses were inoculated to young *N. benthamiana* and zucchini squash (*Cucurbita pepo* cv. Black Beauty).

Northern blot analysis of progeny virus. To extract total RNA, systemically infected leaves of zucchini squash were sampled by taking six leaf discs (ca. 50 mg). The sampled leaf discs were ground in 300 μL of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 2% sodium dodecyl sulfate, and 0.5% 2-mercaptoethanol. The samples were then extracted with phenol treatment, and RNA was precipitated by standard protocol (Sambrook et al., 1989). Total RNAs were fractioned by electrophoresis on 1.6% agarose-formaldehyde denaturing gel and transferred to positively charged nylon membrane (Roche) by capillary method. The membrane was cross-linked by UV and hybridized to digoxigenin-labeled RNA probes complementary to the 3' NCR of all Fny-CMV RNAs. Signals were detected using DIG Luminescent Detection Kit with CSPD (Roche).

Results and Discussion

Analysis of RNA3 quasispecies in lily and *N. benthamiana*. To determine the quasispecies of CMV lily isolates, RT-PCR was performed for the RNA3 derived from systemic leaves of native lily, *N. benthamiana*, and from purified viruses with virusspecific primers. The amplified RT-PCR products were approximately 1.3 kb in length including 3a gene, intergenic region, and 5'-flanking gene of CP. Each product was digested by *Bam*HI enzyme and all the enzyme-treated products of RNA3 showed that they were divided into two groups. The first group in the IR of RNA3 contained recognition sequences of *Bam*HI (indicated by closed arrow in Fig. 2), while the second group had no target sequences of this enzyme (indicated by open arrow in Fig. 2), compared to that of Fny-CMV. In all repeated experiments, these same results were obtained. Moreover, it

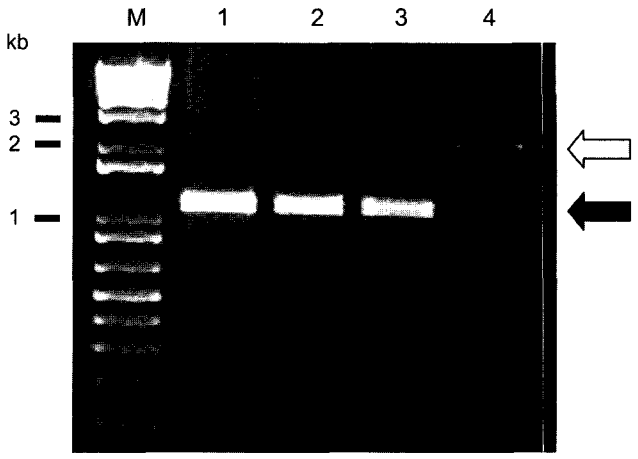

Fig. 2. Detection of quasispecies for LK strain of *Cucumber mosaic virus* (LK-CMV). The RT-PCR products synthesized from cultivated lily, *N. benthamiana* and from purified virus particles using CMV-R3T7 primer and CMV-3ALL primer. Each synthesized product was digested by *Bam*HI enzyme and separated on 1.6% agarose gel. M, 1 kb plus DNA ladder (BRL); 1, lily plant infected by LK-CMV; 2, *N. Benthamiana* infected by LK-CMV; 3, purified virions of LK-CMV; 4, Fny-CMV as control. Open arrow indicates full-length-RNA3 RT-PCR products and closed arrow indicates digested RT-PCR products by *Bam*HI. Molecular weight of size marker in kilobases is marked adjacent to the panel.

was also apparent that the LK strain of CMV was heterogeneous, even after passage through local lesions, as well as through infected plants with each passage through systemic host (data not shown). These results indicate that RNA3 from CMV lily isolates existed in native cultivated lily plants and systemic host plants, as subpopulation. Moreover, these quasispecies of RNA3 were detected from purified virus particles (Fig. 2), indicating that these CMV populations could be transmitted to other plants by vector considering wide host-range of CMV together with analysis of CP sequences (data not shown) (Carrere et al., 1999; Liu et al., 2002).

To examine the population proportion of RNA3 for lily isolates, the 1.3 kb synthesized RT-PCR products were directly cloned into pGEM-T easy vector, and then screened by *Bam*HI digestion. As each clone represented a unique viral RNA, the number of viral clones was used as indicators of population diversity. As shown in Table 1, the RNA3, containing recognition site of *Bam*HI, were dominant in viral populations. These frequencies of RNA3 populations showed similar levels and tendency among isolates, however, the generation proportion of quasispecies in lily plant was larger than those in *N. benthamiana* and purified virus particles (Table 1). Also, RNA3 sub-populations were detected in all the tested plants and purified virions, suggesting that the quasispecies of CMV

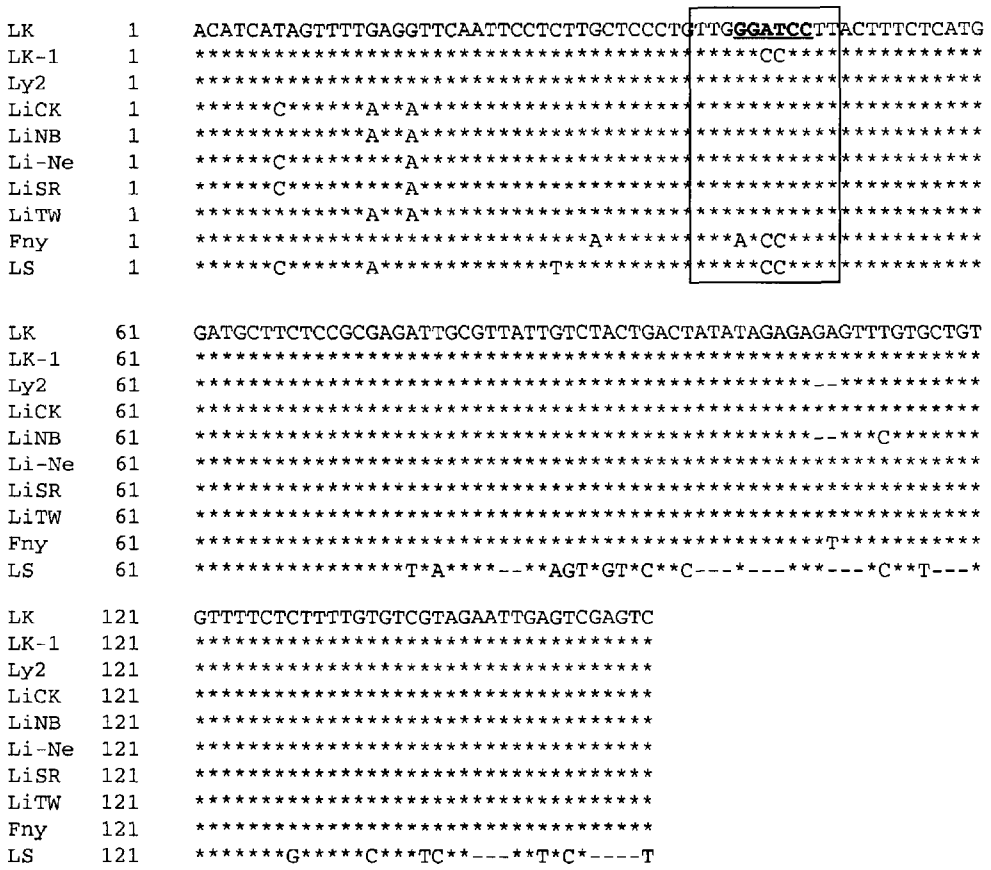

Fig. 3. Multiple sequence alignment analysis of intergenic region (IR) of RNA3 between CMV lily isolates and representative CMV strains. The conserved and absent sequences of IR are represented by asterisk and dash, respectively. The bold letters in the box indicate recognition sequences of *Bam*HI. Two subpopulations of LK-CMV were designated as LK containing *Bam*HI site, and LK-1 without this site. The number of sequence is shown from left with: 1 corresponding to nucleotide (nt) 1106 of LK- CMV RNA3 (Genbank accession number: AJ495841), nt 1102 of Fny RNA3 (D10538), and nt 1087 of LS-CMV (AF127976). The accession numbers for the corresponding sequences of RNA3 of CMV are as follows: Ly2 (AJ296154), LiCK (AJ131616), LiNB (AJ131618), LiSR (AJ131617), and LiTW (AJ131619). The box represented variations of determined nucleotide sequences.

lily isolate frequently occurred in natural conditions.

In addition, nucleotide sequences represented by IR derived from two subpopulations of LK-CMV were aligned with other lily isolates, as well as representative strains of CMV, showing that IR sequences of all the CMV isolates were highly conserved among the same subgroups. Interestingly, in contrast with Fny-CMV and LS-CMV, cDNAs of all CMV lily isolates contained the sequences of *Bam*HI enzyme without exception, and these sequences were changed from C to A, and from C to T (Fig. 3, underlined and bold letters in box). Meanwhile, sequence analysis of RNA3 subpopulations without the *Bam*HI site was shown to preserve the same sequences of two representative CMV strains (Fig. 3).

Results of this study are consistent with previous reports that certain virus-host combinations or environmental effects appeared to contribute to increased variation of the virus (Canto et al., 2001, Chen et al., 2002; Domingo et al., 1996). For CMV and *Tobacco mosaic virus* (TMV), both viruses showed marked variations depending on host changes, even within the same genus hosts. However, diversity of CMV was larger than that of TMV, as extension of host range (Schneider and Roossinck, 2000, 2001). In this category, CMV lily isolates might have a capacity for adaptation via quasispecies for effective movement or viral replication, where virus population rapidly reached the level that was specific to each host plant, and viral RNA populations were adopted in the end (Carrere et al., 1999; Roossinck, 1997; Roossinck and Palukaitis, 1990).

Comparison of biological properties of quasispecies in host plants. To determine if there were differences in pathology between two RNA3 quasispecies, full-length RT-PCR was performed for RNA3 derived from systemic leaf of lily and *N. benthamiana,* as well as from purified virions. Each RT-PCR product was synthesized

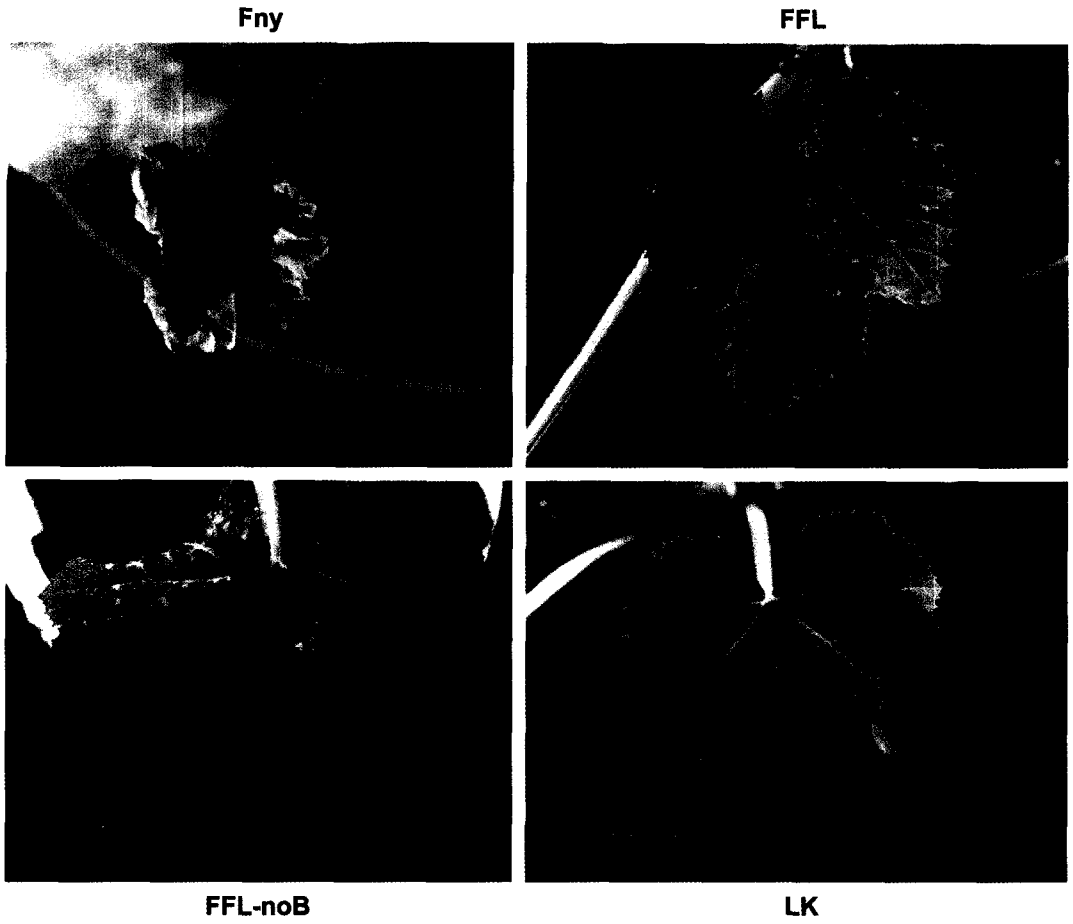

Fig. 4. Systemic symptoms on zucchini squash (cv. Black Beauty) inoculated with two quasispecies RNA3 of LK-CMV and Fny-CMV. The two RNA3 transcripts derived from two cDNA clones LK-CMV were mixed with transcripts RNA1 and 2 of Fny-CMV, generating FFL-CMV without *Bam*HI site and FFL-noB-CMV with *Bam*HI site. Plants infected by two pseudorecombinant CMVs showed chlorotic spot symptom on upper leaves.

approximately 2200bp size using long-template PCR system and cloned into pUC 18. The infectious full-length clones of RNA3s, named as pLK3 (containing *Bam*HI site) and pLK3-noB (no *Bam*HI site), were obtained. RNA3 transcripts with RNA1 and 2 transcripts of cDNA clones of Fny-CMV (designated as FFL and FFL-noB) were inoculated to *N. benthamiana*, and then to zucchini squash (cv. Black Beauty).

In zucchini squash, Fny-CMV induced severe mosaic and stunt symptom of upper leaves of zucchini squash (Gal-On et al., 1995). Meanwhile, LK-CMV did not systemically infect zucchini squash plants, as previously reported (Jung et al., 2000). However, the systemic symptom of zucchini squash infected by FFL-CMV was drastically changed into chlorotic spot at 6~7 dpi (Fig. 4). In the case of zucchini squash infected by FFL-noB-CMV, systemic symptom induced chlorotic spot that was very similar to that of FFL (Fig. 4), but expression time of symptom was slower by 24 hours than that of FFL (data not shown). However, this symptom change showed that the encoded proteins of RNA3 might be more important for the alteration of systemic symptom rather than the variations of IR in the two RNA3 clones. A similar report previously demonstrated that Sny strain of CMV was heterogeneous, even after passage of local hosts. The nucleotide sequences in 3a gene of two cDNA clones derived from Sny-RNA3 that had been isolated from different hosts were highly identical, however, transcripts derived from the two clones showed different phenotype on zucchini squash (Gal-On et al., 1994; Gal-On et al., 1995; Kaplan et al., 1997).

To ascertain whether there was correlation between symptom modification and viral replication, viral RNA accumulations of these viruses were analyzed from the upper leaves of zucchini squash by using Northern blot hybridization. As previously observed for squash infected by Fny-CMV, RNA blot analysis of total nucleic acids extracted from each zucchini squash leaves showed that

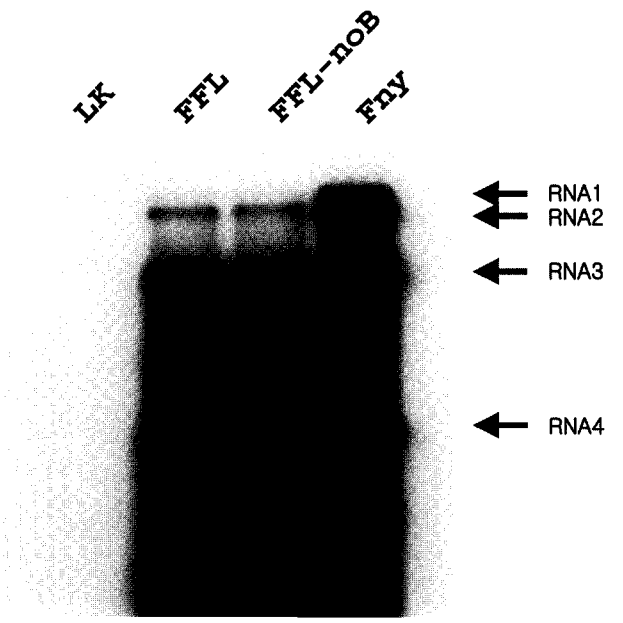

Fig. 5. Northern blot hybridization of viral RNA accumulation in systemic leaves of zucchini squash. All RNA3 transcripts were mixed with transcripts RNA1 and RNA2 from cDNA clones of Fny-CMV, and then inoculated on zucchini squash. The total RNA samples were extracted from systemically infected leaves at 7 days post-inoculation. Fny: wild-type Fny RNA3 generated from full-length infectious clone; pFny309, FFL: RNA3 generated from cDNA clone of LK-CMV that contained *Bam*HI site in IR; FFL-noB: RNA3 derived from cDNA clone of LK-CMV without *Bam*HI site.

viral RNA level for Fny-CMV was higher than two pseudorecombinants (Fig. 5). Furthermore, the level of FFL-CMV RNA was the same as that of FFL-noB RNA, and these pseudorecombinants were able to systemically infect zucchini squash, in contrast to wild-type LK-CMV (Fig. 5). These results indicate that the pathogenicity determinant of LK-CMV on zucchini squash might be located on RNA1 and/or 2 (Gal-On et al., 1994; Roossinck and Palukaitis, 1991). Since zucchini squash plants infected by two pseudorecombinants showed very similar symptom, it appeared that the alteration of symptom severity on zucchini squash might be related to RNA3-encoding proteins, MP and/or CP through virus movement (Canto et al., 1997; Carrere et al., 1999; Kaplan et al., 1997), rather than direct effects of the different IR sequences on both FFL-CMV and FFL-noB-CMV (Boccard and Baulcombe, 1993). Clearly, these results are consistent with previous reports that symptom severity of zucchini squash infected by two pseudorecombinants was correlated with the relatively slower RNA accumulation than Fny-CMV (Roossinck and Palukaitis, 1990). It can be noted that there may be selective factors that generate these quasispecies of viruses, such as changes in the environment, and that development or change of symptom must be further investigated in future researches.

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